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Neuron

Heteromultimerization and NMDA Receptor-Clustering Activity of Chapsyn-110, a Member of the PSD-95 Family of Proteins

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Summary

Chapsyn-110, a novel membrane-associated putative guanylate kinase (MAGUK) that binds directly to N-methyl-D-aspartate (NMDA) receptor and Shaker K⁺ channel subunits, is 70%–80% identical to, and shares an identical domain organization with, PSD-95/SAP90 and SAP97. In rat brain, chapsyn-110 protein shows a somatodendritic expression pattern that overlaps partly with PSD-95 but that contrasts with the axonal distribution of SAP97. Chapsyn-110 associates tightly with the postsynaptic density in brain, and mediates the clustering of both NMDA receptors and K⁺ channels in heterologous cells. Indeed, chapsyn-110 and PSD-95 can heteromultimerize with each other and are recruited into the same NMDA receptor and K⁺ channel clusters. Thus, chapsyn-110 and PSD-95 may interact at postsynaptic sites to form a multimeric scaffold for the clustering of receptors, ion channels, and associated signalling proteins.

Introduction

The proper distribution of voltage-gated and ligand-gated ion channels on the neuronal surface is critical for the processing and transmission of electrical signals in neurons. Different classes of these ion channels are targeted to and largely immobilized at specific sites on the neuronal membrane. The molecular mechanisms that mediate the sorting, clustering, and anchoring of channel proteins is largely unknown, especially in the central nervous system (Froehner et al., 1993).

Recently, the PSD-95/SAP90 subfamily of membrane-associated putative guanylate kinases (abbreviated MAGUKs) has been shown to bind directly to Shaker-type K⁺ channel and N-methyl-D-aspartate (NMDA) receptor 2 (NR2) subunits (Kim et al., 1995; Kornau et al., 1995; Niethammer et al., 1996). Binding occurs directly between the conserved short peptide motif (-ET/SXV) at the C-terminus of the cytoplasmic tails of these subunits and the N-terminal two PDZ domains of PSD-95. In the case of Shaker-subtype voltage-gated K⁺ channels, this interaction results in the coclustering of both channel and PSD-95 proteins in cotransfected cells (Kim et al., 1995). Clustering of NMDA receptors has not yet been demonstrated in heterologous cells, but examples of

PSD-95 colocalization with K⁺ channels and NMDA receptors in neurons have been observed at presynaptic and postsynaptic sites, respectively (Kim et al., 1995; Kornau et al., 1995). Thus, the PSD-95 subfamily of MAGUKs may function as ion channel clustering proteins at synapses and other sites of membrane specialization on the neuronal surface. Mutations of discs large, a Drosophila homolog of PSD-95 (Woods and Bryant, 1991), disrupt normal synaptic structure (Lahey et al., 1994), supporting the idea that the PSD-95 family plays an important role in the molecular organization of synapses in vivo.

The specificity of binding of NR2 and Shaker-type subunits to different members of the PSD-95 family appears to be indistinguishable by in vitro binding and yeast two-hybrid assays (Kim et al., 1995; Niethammer et al., 1996), but the in vivo specificity of interactions between these groups of proteins is unknown. This is an especially significant question since the two characterized members of the PSD-95 family, PSD-95 (also known as SAP90) and SAP97 (also known as hdlg), show rather contrasting subcellular distributions in neurons. PSD-95 has been localized to both presynaptic and postsynaptic locations (Cho et al., 1992; Kistner et al., 1993; Hunt et al., 1996), whereas SAP97 is concentrated in axons and axon terminals (Müller et al., 1995). Shaker-type K⁺ channels are found predominantly in presynaptic terminals and on axons in rat brain, and a subset including Kv1.1 and Kv1.2 are clustered in the juxtaparanodal regions of the nodes of Ranvier (Sheng et al., 1992, 1993, 1994b; Wang et al., 1993, 1994; Maletic-Savatic et al., 1995; Rhodes et al., 1995). In contrast, NMDA receptors are concentrated at postsynaptic sites, though a significant fraction appears to be presynaptic (Aoki et al., 1994; Liu et al., 1994; Petralia et al., 1994; Siegel et al., 1994). Although examples of colocalization of PSD-95 with Shaker-type K⁺ channels have been described in brain (Kim et al., 1995), and with NMDA receptors in cultured neurons (Kornau et al., 1995), the protein expression patterns of PSD-95 and SAP97 in the brain do not overlap completely with the distribution of NMDA receptors and Shaker subfamily K⁺ channels, suggesting the existence of additional members of the MAGUK family that might associate with K⁺ channels and NMDA receptors in the CNS.

Recently, by use of the yeast two-hybrid system, we have identified a third and novel member of the PSD-95 subfamily of MAGUKs that binds to the C-terminus of Shaker K⁺ channel subunits (Kim et al., 1995), as well as to NR2 subunits (Niethammer et al., 1996). We report the cloning and characterization of this protein, which we call chapsyn (channel-associated protein of synapses)-110, and compare its distribution and clustering behavior with its two known relatives, PSD-95 and SAP97. The chapsyn-110 protein is 70%–80% identical to PSD-95 and SAP97 at the amino acid level, and shows an identical domain organization, with three PDZ domains in the N-terminal half, a guanylate kinase (GK)
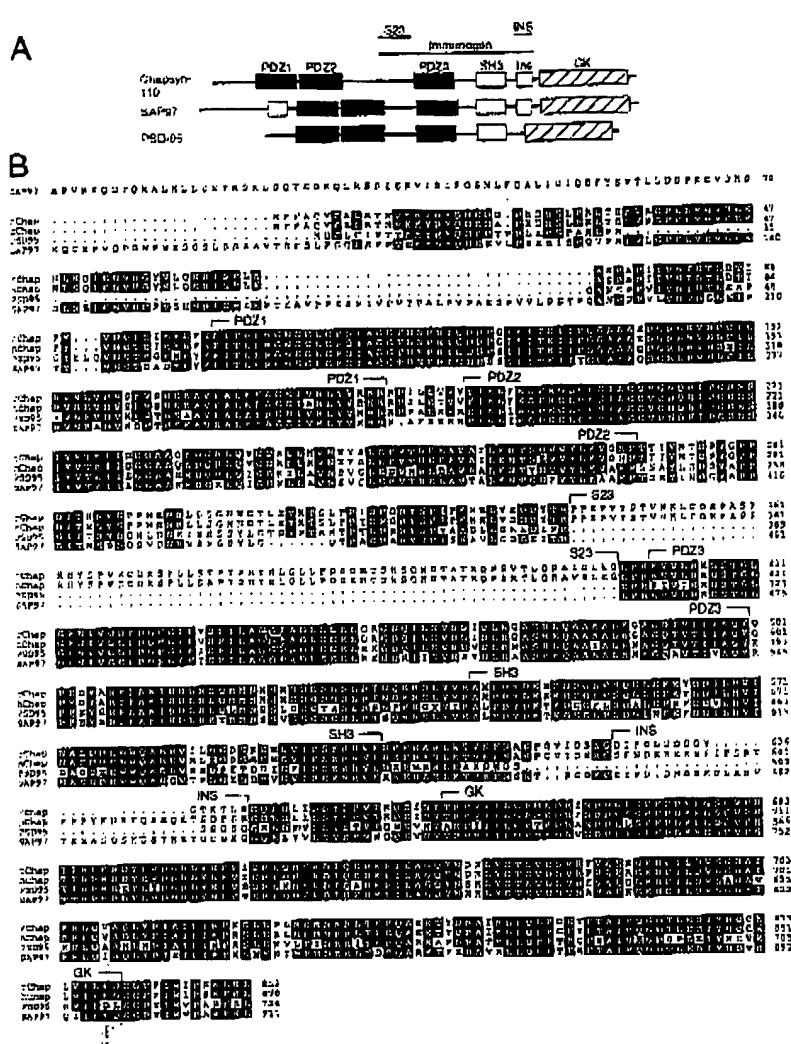
A

B

Figure 1. Structure of Chapsyn-110 Protein

(A) Comparison of the structure and domain organization of chapsyn-110, SAP97, and PSD-95 in schematic form (drawn to scale). White boxes indicate positions at which different sequence insertions aro found, presumably resulting from alternative splicing. The extent of the fusion proteins used for immunization, and for affinity purification of chapsyn-110 antibodies, is indicated (for details, see Results and Experimental Procedures).

(B) Amino acid sequence alignment of chapsyn-110 (rat and human), PSD-95, and SAP97. Residues conserved in at least three of those sequences are shown against a black background, while conservative changes are shaded gray. The boundaries of tho PDZ, SH3, and GK domains are indicated, as are the extents of the Chapsyn-S23 and Chapsyn-INS fusion proteins used for affinity purification of chapsyn-specific antibodies (see Experimental Procedures). Shown at the insertion site (INS) between the SH3 and GK domains are two different sequences for the rat and human genes. Either insertion 1 (shown in the human chapsyn-110 sequence) or insertion 2 (shown in the rat sequence) can be found at this site in chapsyn-110 cDNAs. The rat chapsyn-110 sequence is 97% identical to another rat sequence deposited in GenBank (accession number U50717) termed PSD-93 (Brenman et al., 1996).

homology region of unknown function near the C-terminus, and an intervening SH3 domain. Of particular interest, we show that chapsyn-110 can form heteromultimers with PSD-95, and that both these proteins can cluster NMDA receptor subunits, as well as Shaker type K⁺ channels in heterologous cells.

Results

Primary Structure of Chapsyn-110

A fragment of a novel member of the PSD-95 family (termed "clone 5" in Kim et al., [1995]) was originally isolated in a yeast two-hybrid screen of a human brain cDNA library, using Kv1.4 C-terminal tail as bait. Clone 5 was used to isolate further overlapping cDNAs by hybridization screening of human brain cDNA libraries. The deduced sequence of the full-length cDNA open reading frame predicts a protein of 870 amino acids and a molecular weight of ~95 kD (Figure 1), which is closely related to SAP97 (78% identity, 89% similarity), PSD-95 (71% identity, 85% similarity), and the Drosophila discs large protein (57% identity, 72% similarity). We termed this novel protein chapsyn-110 (apparent molecular size

110 kD) (see below), by analogy to 43K/rapsyn, the acetylcholine receptor clustering molecule of neuromuscular junctions (Apel and Merlie, 1995). The rat gene for chapsyn-110 was independently isolated by low stringency screening of a rat cDNA library using PSD-95, and is ~97% identical to the human homolog at the amino acid level (Figure 1).

The amino acid sequence of chapsyn-110 is most divergent from PSD-95 and SAP97 in three different regions of the protein: the N-terminal region before the first PDZ domain (PDZ1); the region between PDZ2 and PDZ3; and the segment between the SH3 and guanylate kinase domains. In this inter-SH3/GK region we have found evidence of two alternative insertions (of length 33 and 15 amino acids) occurring after residue 626 of chapsyn-110, presumably reflecting differential exon splicing at this site. Interestingly, hdlg/SAP97 also exhibits alternative exon usage (Lue et al., 1994) in the same region between its SH3 and GK domains.

Northern analysis revealed that chapsyn-110 mRNA is expressed predominantly in neural tissues (Figure 2). There were two transcripts (major band ~6.0 kb, minor band ~5.0 kb) expressed in brain, and to a lesser extent,
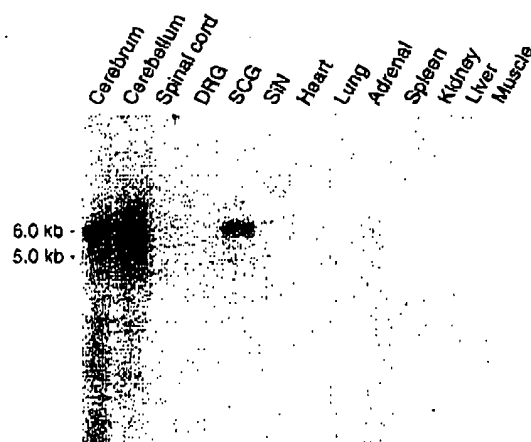
Figure 2. Northern Blot Analysis of Chapsyn-110 mRNA Expression in Multiple Rat Tissues

10 μg of total RNA from the indicated tissues were probed with chapsyn-110 cDNA at high stringency. DRG: dorsal root ganglia; SCG: superior cervical ganglion, SiN: sciatic nerve.

in superior cervical ganglion. Chapsyn-110 mRNA was not detected in heart, skeletal muscle, kidney, lung, liver, or spleen, but a faint signal was observed in the adrenal gland.

Specificity of Chapsyn-110, PSD-95, and SAP97 Antibodies

To investigate chapsyn-110 expression at the protein level, chapsyn-110-specific antibodies were generated. Rabbit antisera were raised against a bacterial fusion protein incorporating chapsyn-110 residues 343–660, which begins in the middle of the region between PDZ2 and PDZ3, and that extends to include insertion 1 located between SH3 and GK domains (Figure 1). To derive antibodies specific for chapsyn-110, which do not cross-react with other PSD-95 family members, these antisera were then affinity purified, using a fusion protein encompassing residues 343–417 (termed Chapsyn-S23) corresponding to the highly divergent spacer region between PDZ2 and PDZ3 and a fusion protein incorporating residues 627–660 (termed Chapsyn-INS), corresponding to the unique insertion between SH3 and GK domains (insertion 1, Figure 1B). Chapsyn-S23 antibodies should recognize all forms of chapsyn-110, whereas Chapsyn-INS antibodies would be expected to recognize the subset of chapsyn-110, which contains insertion 1. The majority of the chapsyn-110 cDNAs we have isolated contain the insertion 1 sequence.

Chapsyn-S23 antibodies specifically recognize a diffuse band of Mr ~110 kD in immunoblots of chapsyn-110-transfected COS-7 cells, but do not cross-react with SAP97 or PSD-95-transfected cells by immunoblotting (Figure 3A), or by immunocytochemistry (data not shown). By similar analysis, the PSD-95 and SAP97 antibodies recognize their respective antigens, but show no cross-reactivity with the other two members of the family by immunoblotting or immunostaining of transfected COS-7 cells (Figure 3A; data not shown).
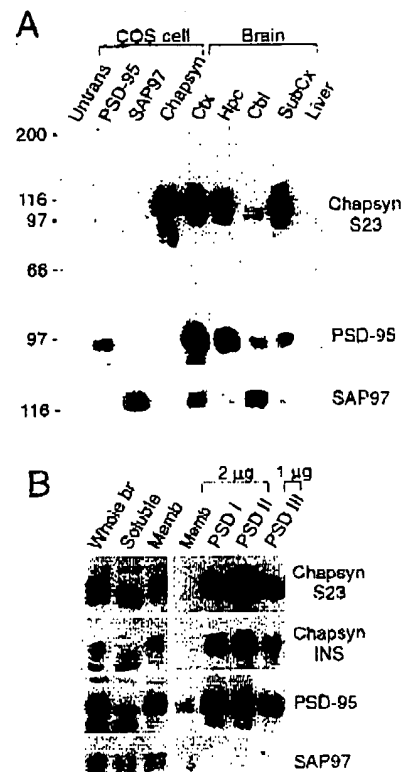

Figure 3. Expression of Chapsyn-110 Protein in Rat Brain

(A) Immunoblot analysis of specificity of chapsyn-110, PSD-95, and SAP97 antibodies and differential regional expression of these proteins in rat brain. Whole cell extracts of untransfected COS-7 cells, and of cells transfected with PSD-95, SAP97, or chapsyn-110 cDNAs, were separated on a 6% polyacrylamide/SDS gel along with membrane fractions (10 μg protein) from different regions of brain or liver, as indicated. Filters were probed with Chapsyn-S23, PSD-95, and SAP97 antibodies, as indicated. Positions of molecular size markers are shown in kD. Abbreviations: Untrans. (untransfected), Ctx (cortex), Hpc (hippocampus), Cbl (cerebellum), Subcx (subcortical regions).

(B) Differential subcellular fractionation of chapsyn-110, PSD-95, and SAP97. Lanes were loaded with rat brain fractions, as follows: whole br (total brain homogenate, 20 μg protein), soluble (S100 supernatant fraction of brain homogenate, 30 μg), memb (crude synaptosomal membrane fraction, 10 μg and 2 μg); PSDI, PSDII, PSDIII (purified PSD fractions after extraction with Triton X-100 once (I), twice (II), or with Triton X-100 followed by sarkosyl (III)). Filters were probed with Chapsyn-S23, Chapsyn-INS, PSD-95, and SAP97 antibodies, as indicated. Different quantities of protein were loaded into the lanes: to show relative fractionation, equal percentages (rather than equal mass) of membrane and soluble fractions were loaded; the soluble fraction contained three times higher concentration of total protein than the membrane fraction. To show purification into the PSD fractions, only 2 μg of PSDI, PSDII, and 1 μg of PSDIII were immunoblotted, and compared with 2 μg of membrane fraction. Due to nonlinearity of the chemiluminescent reaction and of the photographic process, the weak chapsyn-110 signal in 2 μg of crude synaptosomal membranes became almost invisible.

Differential Regional Expression and Subcellular Fractionation of Chapsyn-110 in Rat Brain

On immunoblots of rat brain membranes, Chapsyn-S23 antibodies specifically recognize a heterogeneous band of ~110 kD that comigrates with the band found in
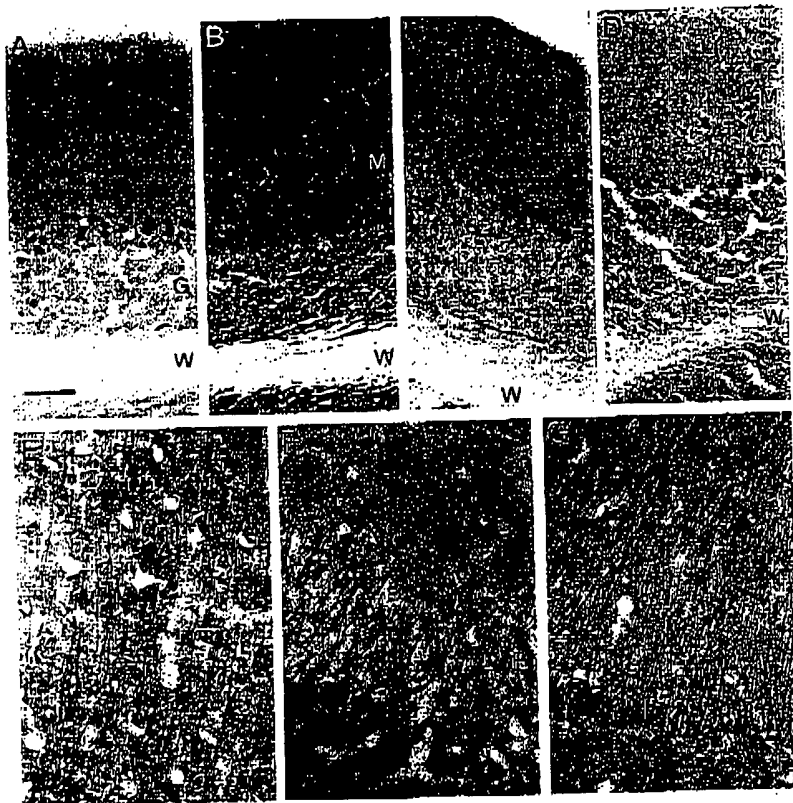
Figure 4. Differential Immunohistochemical Localization of Chapsyn-110 in Rat Brain

(A–D) Immuno-staining of cerebellar cortex showing contrasting patterns of immunoreactivity for chapsyn-110 (A and D), SAP97 (B), and PSD-95 (C). Note the similar somatodendritic staining of Purkinje cells with two different antibodies to chapsyn-110: Chapsyn-S23 (A) and Chapsyn-INS (D). Abbreviations: M, molecular layer; P, Purkinje cell layer; G, granule cell layer; W, white matter.

(E–G) Somatodendritic localization of chapsyn-110, as detected by Chapsyn-S23 antibodies, in neurons of cerebral cortex layer 5 (E), pyramidal neurons of area CA3 (F) and of area CA1 (G) of hippocampus. Abbreviations: P, pyramidal cell layer; Sr, stratum radiatum. Scale bar represents 200 μm (A–D), 100 μm (E–G).

COS-7 cells transfected with chapsyn-110 cDNA (Figure 3A). Chapsyn-110 protein is abundant in membrane fractions from cerebral cortex, hippocampus, and subcortical regions, but is present at lower levels in cerebellum. A similar size band and expression pattern was seen using the chapsyn-INS antibodies (data not shown). The regional expression pattern of chapsyn-110 is somewhat similar to PSD-95, but markedly different from SAP97, which is most abundant in cerebellum and at relatively low levels in hippocampus and subcortical regions (Figure 3A).

Like PSD-95 and SAP97, chapsyn-110 is associated with the crude synaptosomal membrane fraction from rat brain (Figure 3B). A significant proportion of all three, however, is found in the soluble fraction, which is particularly enriched for the lower molecular weight components of the chapsyn-110 and PSD-95 immunoreactive bands (Figure 3B). These apparently lower molecular weight soluble species may represent immaturely processed forms of chapsyn-110 and PSD-95. Since none of these proteins contain predicted transmembrane domains, they are presumably peripheral membrane proteins. Membrane-associated chapsyn-110 further purifies into the postsynaptic density fraction where it is resistant to both Triton X-100 and sarkosyl detergent extraction (Figure 3B). In this respect, its behavior resembles that of PSD-95 (Figure 3B; Cho et al., 1992) and contrasts with that of SAP97. SAP97 is associated with synaptosomal membranes but does not copurify with PSD-95 or chapsyn-110 into postsynaptic density fractions (Figure 3B); this finding is consistent with SAP97 being predominantly an axonal and nerve terminal protein (Müller et al., 1995).

Immunohistochemical Distribution of Chapsyn-110 Protein in Rat Brain

Affinity purified Chapsyn-S23 and Chapsyn-INS antibodies were used to localize chapsyn-110 protein in rat brain sections. Chapsyn-S23 immunostaining was consistently stronger, though the patterns of immunoreactivity were similar with both antibodies (Figure 4A and 4D).

Chapsyn-110 protein is widely expressed in neurons throughout the brain, but at particularly high levels in cortex, hippocampus, and basal ganglia. At the subcellular level, chapsyn-110 immunoreactivity was distributed consistently in a somatodendritic pattern, with light staining of the cell bodies and prominent staining of dendrites and surrounding neuropil, clearly seen in cortex and hippocampus (Figures 4E, 4F, and 4G). The somatodendritic staining pattern of chapsyn-110 in hippocampal pyramidal neurons is similar to that reported for PSD-95 (Cho et al., 1992).

The immunostaining pattern of chapsyn-110 is distinct, however, from that of PSD-95 or SAP97. The differential distribution of chapsyn-110 with respect to PSD-95 and SAP97 is most clearly seen in the cerebellum, where chapsyn-110 immunoreactivity is prominently found in the soma and dendritic trees of Purkinje cells (Figure 4A). In contrast, SAP97 immunostaining is strongest in the neuropil of the molecular layer, and the Purkinje cell bodies and dendrites stand out in relief as unstained elements (Figure 4B). This cerebellar SAP97 staining pattern is in agreement with Müller et al. (1995) who showed SAP97 to be concentrated in parallel fiber axons. PSD-95 immunoreactivity shows a third distinct pattern, being particularly highly concentrated in the
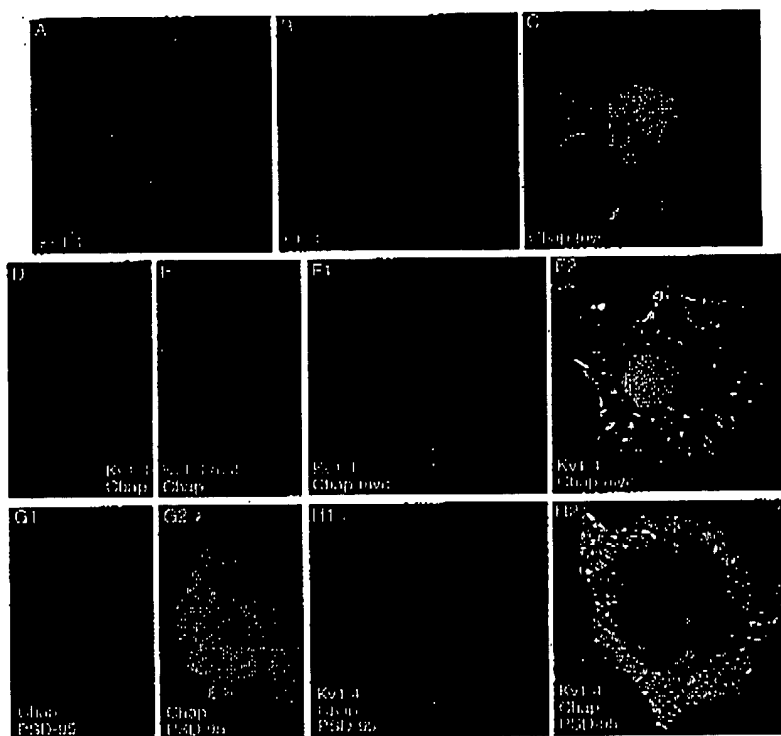
Figure 5. K⁺ Channel Clustering Activity of Chapsyn-110

COS-7 cells were transfected with cDNAs encoding the proteins listed at the bottom left of each panel. When multiple proteins are coexpressed, the name of the protein that is visualized by the primary antibody is indicated in color (red or green indicates that the secondary antibody was labeled with Cy3 or FITC, respectively). (A), (B), and (C) indicate different cells visualized, while F1 and F2 indicate the same cell visualized through different immunofluorescence channels. When individually expressed in COS-7 cells, Kv1.4 (A), chapsyn-110 (B), and myc-tagged chapsyn-110 (C) are diffusely distributed in the cell. Coexpression of chapsyn-110 results in the clustering of Kv1.4 (D), but not of a Kv1.4 mutant in which the C-terminal sequence has been changed from -ETDV to -ETDA (E). Coexpression of Kv1.4 and myc-tagged chapsyn-110 results in the coclustering of both proteins, as visualized by double immunofluorescence in the same cell by Kv1.4 antibodies (F1, red), and 9E10 anti-myc mouse monoclonal antibody (F2, green). Since the anti-myc signal (green) was lower than the Kv1.4 signal (red), the photographic process resulted in the false impression that the myc-chapsyn-110 clusters are smaller than the Kv1.4 clusters. When viewed directly, those two proteins are exactly colocalized. In cells coexpressing chapsyn-110 and PSD-95, both chapsyn-110 (G1, red) and PSD-95 (G2, green) show indistinguishable diffuse distributions throughout the cell. In cells triply transfected with Kv1.4 + chapsyn-110 + PSD-95, typical clusters form in which chapsyn-110 (H1, red) and PSD-95 (H2, green) are exactly colocalized by double immunofluorescence. Abbreviations: Chap (chapsyn-110); Chap-myc (myc epitope-tagged chapsyn-110); Kv1.4 mut (Kv1.4 -ETDA C-terminal mutant).'

basket cell nerve terminal plexuses wrapped around the axon hillocks of Purkinje neurons (Figure 4C). Taken together with immunoblotting data, these results indicate that chapsyn-110, PSD-95, and SAP97 proteins show distinctive (though in the case of chapsyn-110 and PSD-95, overlapping) patterns of expression at the regional, cellular, and subcellular levels in rat brain.

Chapsyn-110 Is a K⁺ Channel Clustering Molecule

To test whether chapsyn-110 can function like PSD-95 in ion channel clustering (Kim et al., 1995), chapsyn-110 was cotransfected into COS-7 cells with the Shaker-type K⁺ channel subunit Kv1.4. Expressed by itself, chapsyn-110 immunoreactivity is diffusely distributed in COS cells (Figure 5B); Kv1.4 by itself shows a diffuse surface distribution and an intracellular immunoreactivity pattern suggestive of accumulation in the endoplasmic reticulum, in agreement with previous studies (Figure 5A; Kim et al., 1995). When cotransfected in the same cells, however, chapsyn-110 results in the dramatic surface clustering of Kv1.4 (Figure 5D), but not of a Kv1.4 mutant in which the C-terminal binding motif had been mutated from -ETDV to -ETDA (Figure 5E). Coclustering of the chapsyn-110 and wild-type Kv1.4 could not be directly shown by double immunofluorescence because antibodies to both proteins were derived from rabbits. Therefore, a myc epitope-tagged version of chapsyn-110 was constructed. Cotransfection experiments with chapsyn-110-myc produced Kv1.4 -channel clustering

as with wild-type chapsyn-110, but by using mouse monoclonal antibodies (9E10) recognizing the myc-epitope, we could demonstrate that in coexpressing cells, chapsyn-110 coclustered with Kv1.4 in the same plaques at the cell surface (Figure 5F). The size, shape, and texture of chapsyn-110/Kv1.4 coclusters is very similar to those seen with PSD-95/Kv1.4 (Kim et al., 1995; data not shown). Thus, chapsyn-110 behaves like PSD-95 not only in its ability to bind Shaker subfamily and NR2 subunits (Kim et al., 1995; Niethammer et al., 1996), but also in its ability to cocluster with Shaker K⁺ channels.

Chapsyn-110 Is Recruited into the Same K⁺ Channel Clusters as PSD-95

COS cells doubly transfected with chapsyn-110 and PSD-95 show a diffuse intracellular immunoreactivity for both proteins, similar to the pattern seen with either protein alone (Figure 5G). Thus, these two MAGUKs do not induce each other to aggregate or cluster when coexpressed. In triple (Kv1.4 + PSD-95 + chapsyn-110) transfected cells, however, typical surface clusters were formed in which both PSD-95 and chapsyn-110 were exactly colocalized (Figure 5H). In parallel experiments, these chapsyn-110/PSD-95 containing clusters were shown to colocalize with Kv1.4 immunoreactivity (data not shown). These results imply that Kv1.4 recruits both chapsyn-110 and PSD-95 into the same membrane-associated complexes, at least at the light microscopic level.
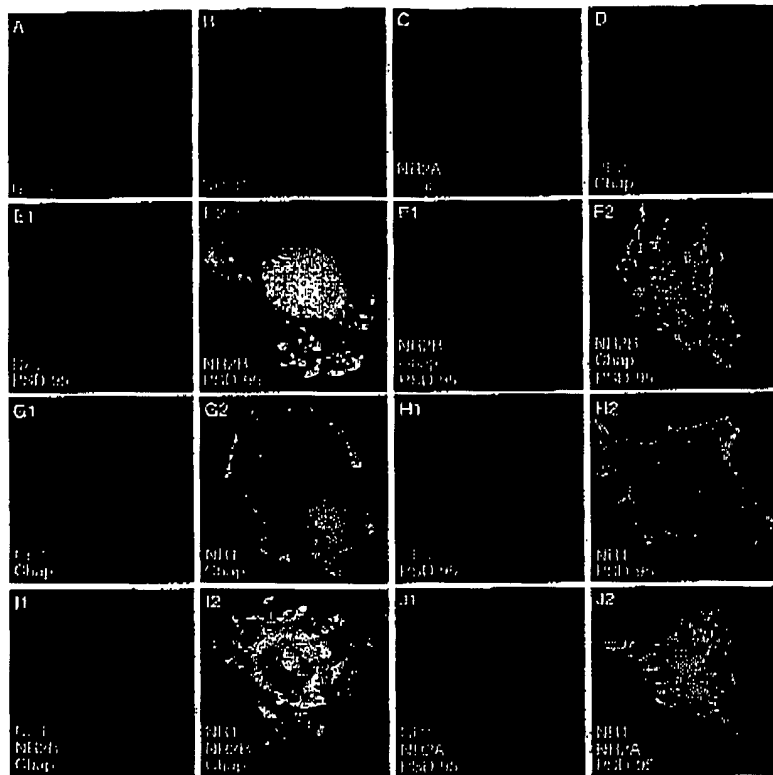
Figure 6. NMDA Receptor Clustering Activity of Chapsyn-110 and PSD-95

Panels are labeled using the same scheme as in Figure 5. When individually expressed in COS-7 cells, NR2A (A) and NR2B (B) are distributed in a perinuclear and cytoplasmic reticular pattern. Cotransfection of chapsyn-110 and NR2A results in redistribution of both chapsyn-110 (C) and NR2A (D) into plaque-like clusters. Similarly, coexpression of NR2B and PSD-95 results in clusters in which NR2B and PSD-95 can be directly colocalized in the same cell (E1, E2). Chapsyn-110 and PSD-95 behaved identically with respect to clustering of either NR2A or NR2B (data not shown). Cells triply transfected with NR2B + chapsyn-110 + PSD-95 form typical clusters in which chapsyn-110 and PSD-95 are exactly colocalized by double immunofluorescence (F1, F2). In cells doubly transfected with NR1 and chapsyn-110 (G1, G2), or NR1 and PSD-95 (H1, H2), both proteins are diffusely distributed with no evidence of colocalization. However, in cells triply transfected with NR1 + NR2A or NR2B + chapsyn-110 or PSD-95, NR1 colocalizes in clusters with chapsyn-110 (I) and PSD-95 (J). The NR2B antibodies gave some background nuclear staining. Abbreviations: NR1 (NR1A splice variant; Sugihara et al., 1992).

Clustering of NMDA Receptors by Chapsyn-110 and PSD-95

Although PSD-95 and its relatives also bind specifically to the C-terminal tails of NR2 subunits, clustering of NMDA receptors by these proteins has not been previously shown. When NR2A or NR2B is transfected alone into COS-7 cells, the expressed protein is distributed in a perinuclear and a cytoplasmic reticular pattern (Figures 6A and 6B) not dissimilar from that of Kv1.4 (Figure 5A). Cotransfection with chapsyn-110 or PSD-95, however, results in the formation of plaque-like clusters of NR2A (Figure 6D) and NR2B (Figure 6E1), in which PSD-95 can be shown to be colocalized (Figure 6E2). The appearance of these coclusters is essentially indistinguishable from that of Kv1.4 clusters, although the efficiency of NR2 clustering (~10%–20% of cotransfected cells show convincing cluster formation) is lower than that of Kv1.4 clustering (~40%–70%), possibly due to the weaker expression level of NR2 proteins. In triple transfections of NR2 + chapsyn-110 + PSD-95, typical plaque-like clusters were formed in which chapsyn-110 and PSD-95 exactly colocalize (Figures 6F1 and F2); thus NR2A and NR2B behave like Kv1.4 in being able to recruit both chapsyn-110 and PSD-95 into the same clusters. Identical results were obtained with either NR2A or NR2B in these clustering experiments, so only representative results are shown. For either K channel or NMDA receptor subunits, we found no significant difference in the relative clustering efficiencies of the homomeric chapsyn-110 or PSD-95 versus the heteromeric PSD-95/chapsyn configurations.

Formation of fully functional NMDA receptors requires the NR1 subunit, in addition to NR2 subunits (Meguro

et al., 1992; Monyer et al., 1992). In contrast with NR2, we find that expression of the NR1 subunit (the common splice variant NR1A) with either chapsyn-110 or PSD-95 does not result in cocluster formation (Figures 6G and 6H). Instead, both proteins remain diffusely distributed in the same cell as if each was individually expressed, consistent with this NR1 subunit not having the C-terminal binding motif for PDZ domains. Significantly, however, in triple transfections with NR1 + NR2A/NR2B + chapsyn-110/PSD-95, NR1 did cocluster with chapsyn-110 and PSD-95 (Figure 6I and 6J). The requirement for NR2 subunit coexpression in NR1 clustering implies that NR1/NR2 heteromeric NMDA receptors were formed that then clustered via the binding of chapsyn-110 and PSD-95 to the NR2 subunits. Taken together, these results demonstrate that chapsyn-110 and PSD-95 can cluster heteromeric NMDA receptors, as well as NR2 subunits.

Chapsyn-110 Heteromultimerizes with PSD-95 but Not SAP97

The coclustering of chapsyn-110 and PSD-95 in the presence of Kv1.4 or NR2 subunits could reflect recruitment of chapsyn-110 and PSD-95 monomers, chapsyn-110, and PSD-95 homomultimers, or chapsyn-110/PSD-95 heteromultimers, into the clusters. To test whether chapsyn-110 and PSD-95 can form heteromeric complexes, these two proteins were expressed together with SAP97 in COS-7 cells in the absence of channel subunits, and their association was assayed by coimmunoprecipitation. Control experiments indicated that chapsyn-S23 and SAP97 antibodies are highly specific for their respective antigens in immunoprecipitation from
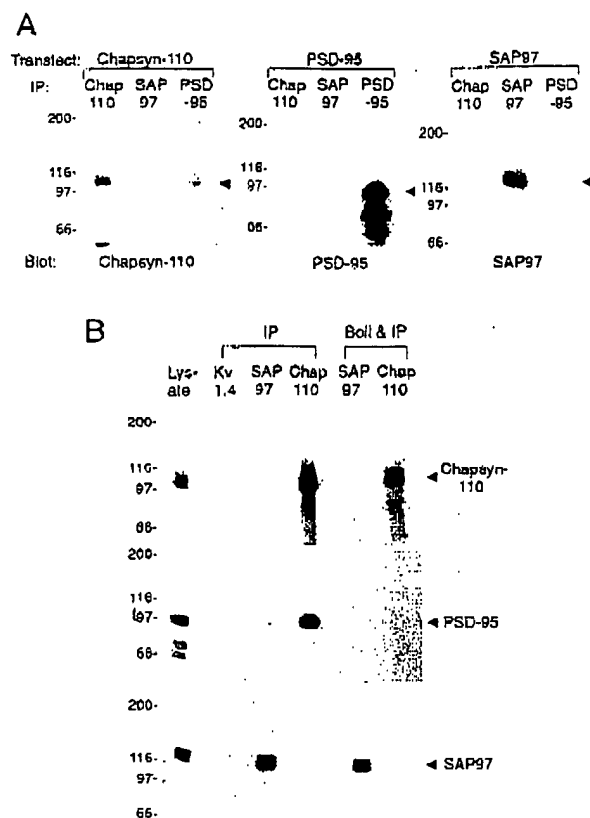
A

B

Figure 7. Coimmunoprecipitation of Chapsyn-110 and PSD-95

(A) Specificity of chapsyn-110 and SAP97 antibodies in immunoprecipitation. COS-7 cells were separately transfected with chapsyn-110, SAP97, or PSD-95 cDNAs, and equal amounts of each cell lysate were immunoprecipitated ("IP") with Chapsyn-S23, SAP97 or PSD-95 antibodies, as indicated. The immunoprecipitates were then immunoblotted for chapsyn-110, SAP97, and PSD-95, as shown. Chapsyn-S23 and SAP97 antibodies are highly specific for their cognate antigens, whereas PSD-95 antibodies shows slight cross-reactivity to chapsyn-110, in this immunoprecipitation assay. Thus, only Chapsyn-S23 and SAP97 antibodies were used as immunoprecipitating antibodies in part B.

(B) Coimmunoprecipitation of chapsyn-110 and PSD-95. Extracts from COS-7 cells triply transfected with chapsyn-110 + PSD-95 + SAP97, were immunoprecipitated with Chapsyn-S23, SAP97 or negative control Kv1.4 antibodies, as indicated. Immunoprecipitates were then immunoblotted for PSD-95, chapsyn-110, or SAP97, as indicated at the right of each panel. The first lane ("lysate") was loaded directly with the transfected cell lysate. In "boil and IP" controls, extracts were first denatured in boiling SDS before immunoprecipitation with the indicated antibodies.

singly transfected COS-7 cell extracts (Figure 7A). However, from detergent extracts of triply (PSD-95 + chapsyn-110 + SAP97) transfected cells, chapsyn-S23 antibodies consistently immunoprecipitated large amounts of PSD-95 in addition to chapsyn-110 (Figure 7B). Interestingly, there was no detectable coimmunoprecipitation of SAP97 with chapsyn-110 antibodies, and only weak coimmunoprecipitation of PSD-95 with SAP97 antibodies (Figure 7B), suggesting that chapsyn-110 and PSD-95 associate preferentially with each other rather than with SAP97. Negative control Kv1.4 antibodies did not immunoprecipitate any of these proteins. PSD-95

antibodies showed slight cross-reactivity for chapsyn-110 in immunoprecipitation (Figure 7A), and were therefore not used as immunoprecipitating antibodies in these experiments.

The coimmunoprecipitation of PSD-95 using chapsyn-S23 antibodies is extremely unlikely to represent cross-reactivity of these antibodies for PSD-95 because chapsyn-S23 antibodies are directed against a unique region in chapsyn-110 (Figure 1B); chapsyn-S23 antibodies do not cross-react with PSD-95 or SAP97 on Western blots (Figure 3A) or by immunoprecipitation from singly transfected COS-7 cells (Figure 7A); and coimmunoprecipitation of PSD-95 by Chapsyn-S23 antibodies was abolished by denaturation of COS cell extracts in boiling SDS, while immunoprecipitation of chapsyn-110 was little affected (Figure 7B), suggesting that PSD-95 and chapsyn-110 associate through noncovalent SDS-sensitive bonds. These coimmunoprecipitation results indicate that chapsyn-110 and PSD-95 form heteromeric complexes even in the absence of Kv1.4, and when both proteins are diffusely distributed in the cell (Figure 5G).

Not surprisingly, when cells are quadruply transfected with Kv1.4 plus the three MAGUKs, chapsyn-110 and PSD-95 remain coimmunoprecipitable with each other, but in addition, Kv1.4 is now coimmunoprecipitated with either chapsyn-110 or PSD-95 (data not shown). These results confirm that the coclustering of all three proteins seen in these cells is based on a complex formed between K⁺ channel subunits and chapsyn-110 and PSD-95.

Discussion

Chapsyn, a Novel Member of the PSD-95 Family of Ion Channel Clustering Proteins

Chapsyn-110, a third and new member of the PSD-95 subfamily of MAGUKs, is 70%–80% identical to PSD-95 and SAP97, and approximately as different from PSD-95 and SAP97 as the latter are different from each other. The domain organization is identical in this family, with the major differences in primary structure occurring outside the highly conserved PDZ, SH3, and GK domains. This close sequence homology is reflected by some functional similarities of these proteins. Like its two known relatives, chapsyn-110 binds to the C-terminal tails of Shaker K⁺ channel and NR2 subunits through its highly conserved PDZ1 and PDZ2 domains (Kim et al., 1995; Niethammer et al., 1996). Furthermore, in heterologous cells, chapsyn-110 coexpression results in coclustering with Shaker-type K⁺ channels and NR2 subunits at the cell surface, similar to the coclustering seen with PSD-95 (Kim et al., 1995). Thus, with respect to primary structure, in vitro protein-protein interactions, and channel clustering activity in heterologous cells, chapsyn-110 is homologous to other members of the PSD-95 family. Thus, chapsyn-110 joins an emerging set of proteins that are likely to be important, not only in the clustering and anchoring of ion channels and receptors, but also in the coupling of receptors/ion channels to intracellular signalling pathways, perhaps by acting as scaffolds for a submembrane signalling complex (Brenman et al., 1996). However, an important question is

raised by the multiplicity of genes in this family: do these highly related proteins have different functions in vivo?

Differential Subcellular Localization of Chapsyn-110

A clue to potential differences in function is provided by the differential localization of chapsyn-110 and its relatives in vivo. Chapsyn-110, PSD-95, and SAP97 are widely expressed in distinct though overlapping patterns in rat brain. At the subcellular level, chapsyn-110 distribution shows much in common with that of PSD-95 but is in sharp contrast with SAP97. The somatodendritic localization of chapsyn-110 in cortical and hippocampal pyramidal neurons, for instance, is similar to that described for PSD-95 (Cho et al., 1992; Hunt et al., 1996), and both these proteins copurify with each other in detergent-extracted postsynaptic density fractions. Clearly, however, some fraction of PSD-95 is also found in axon terminals of inhibitory neurons (Kistner et al., 1993; Kim et al., 1995). Thus, PSD-95 can be considered "mixed" pre- and postsynaptic. Chapsyn-110 appears to be a predominantly postsynaptic member of this family; whether some fraction is located presynaptically will require immunoelectronmicroscopic resolution. SAP97, in contrast, is targeted to axons and excitatory presynaptic terminals (Müller et al., 1995), a conclusion that is supported by our independent immunohistochemical data (Figure 4 and E. K. and M. S., unpublished data). Consistent with SAP97 being segregated to the axonal/terminal pole of neurons, our biochemical studies reveal SAP97 to be unique among this family in not being associated with the postsynaptic density. In conclusion, although the expression pattern of chapsyn-110 is distinct from the other two members of the family, its postsynaptic distribution overlaps with PSD-95 most closely.

Functional Similarities between Chapsyn-110 and PSD-95

In addition to similarities in subcellular localization, chapsyn-110 shows functional activities that resemble PSD-95 rather than SAP97. Although all three of these MAGUKs have indistinguishable binding specficities for K^+ channels and NMDA receptors in vitro and in the yeast two-hybrid system, only chapsyn-110 and PSD-95 form coclusters efficiently at the cell surface with channel subunits. SAP97 does interact with Kv1.4 when coexpressed in COS-7 cells, but the two proteins form large intracellular coaggregates (E. K. and M. S., unpublished data) rather than the flat coclusters seen at the cell surface with chapsyn-110 or PSD-95. The reason for this different clustering behavior is unknown. SAP97 may have distinct molecular requirements for surface clustering that are not present in COS-7 cells, and that may reflect the specialization of SAP97 for an axonal function.

Perhaps the most compelling sign that chapsyn-110 and PSD-95 are more closely related functionally is that they form heteromultimers with each other but not with SAP97, and are recruited into the same clusters when coexpressed with K^- channel or NMDA receptor proteins. Furthermore, they both interact with neuronal nitric oxide synthase (Brenman et al., 1996). Given that

there is substantial overlap in their distribution in rat brain, these results suggest that chapsyn-110 and PSD-95 may cooperate at common postsynaptic sites to cluster ion channels and their associated intracellular signaling proteins.

NMDA Receptor-Clustering by Chapsyn-110 and PSD-95

NR2 subunits share the C-terminal ES/TXV motif with Shaker subunits, but are otherwise totally dissmilar from their K^+ channel counterparts. Therefore, successful clustering of NR2 proteins by chapsyn-110 and PSD-95 (Figure 6) reinforces the idea that binding of the C-terminal tail of these membrane proteins is crucial in the mechanism of clustering. Together with colocalization data (Kornau et al., 1995), our results indicate that an interaction between NR2 subunits and chapsyn/PSD-95 in the postsynaptic density may underlie the clustering of NMDA receptors at postsynaptic sites. Moreover, since NR2 subunits bind to either PDZ1 or PDZ2 of chapsyn-110 and PSD-95 (Kornau et al., 1995; Niethammer et al., 1996) and neuronal nitric oxide synthase binds to PDZ2 (Brenman et al., 1996), the clustering activity of chapsyn-110 and PSD-95 should result in the coaggregation of NMDA receptors with a downstream signaling protein (nNOS) that is regulated by NMDA receptor activation. Furthermore, our demonstration of NMDA receptor clustering now make it possible to test whether PSD-95 family proteins can organize heterogeneous ion channel clusters in which K^+ channels and NMDA receptors are intimately mixed, as might be the case in vivo.

The important finding that NR1/NR2 complexes are coclustered with either chapsyn-110 or PSD-95 implies that the PSD-95 family can cluster heteromeric ionotropic receptors as well as homotetrameric ion channels like Kv1.4 K^+ channels. The subunit stoichiometry of NMDA receptors is still unresolved, so it is currently unclear how many ES/TXV-containing subunits per channel are required for clustering by the PSD-95 family of proteins. The simplest cross-linking model (Gomperts, 1996; see below) requires at least two C-terminal ES/TXV binding motifs per channel complex, and this criterion is likely to be fulfilled in heteromeric NMDA receptors. Since NR2 subunits by themselves are clustered by chapsyn-110 and PSD-95, this model raises the intriguing possibility that NR2 subunits might form homo-oligomers, even though these are not functional as NMDA receptors.

Heteromultimerization of Chapsyn-110 and Mechanisms of Ion Channel Clustering

The finding that chapsyn-110 and PSD-95 are associated in a heteromeric complex in the absence of Kv1.4 or NR2 subunits indicates that PSD-95 family proteins can exist as multimers even prior to coclustering with ion channels. The stoichiometry of multimerization, whether individual members of the PSD-95 family also form homo-multimers, and whether they bind directly to each other or via some endogenously expressed linker protein, are important questions that remain to be answered. In view of the similarities in subcellular distribution and clustering activity shared by chapsyn-110 and

PSD-95, it may be especially significant that these two members of the family associate much more efficiently with each other than with SAP97. The data raise the possibility that PSD-95 and chapsyn-110 are closely interacting at postsynaptic sites in vivo. An analogous heteromeric association is known to exist for two tight junction proteins of the MAGUK superfamily, ZO-1 and ZO-2, which can be coimmunoprecipitated from epithelial cells (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994).

Previous models of K^+ channel clustering by monomeric PSD-95 depended on the presence of two PDZ domains per PSD-95 molecule that can bind channel subunits. In these cross-linking models, tetrameric K^+ channels containing four C-terminal tails could be cross-linked into raft-like clusters in the membrane by the divalent binding sites on each PSD-95 molecule (Kim et al., 1995; for review, see Gomperts, 1996). The discovery of PSD-95/chapsyn-110 multimerization offers novel insight into the mechanism of ion channel clustering because it obviates the requirement for multiple channel binding sites per PSD-95 monomer. Instead, a single binding site (such as one of the PDZ domains) on each MAGUK molecule can contribute to channel cross-linking by being part of a multivalent multimeric complex.

The ability of these MAGUKs to associate with themselves or with each other implies a stoichiometric multiplication of the number of PDZ domains in each complex. Since PDZ domains are directly responsible for the binding of membrane ion channels, the multimerization of chapsyn-110 and PSD-95 at specific membrane sites can increase the local concentration of channel binding sites and enhance the ability of these proteins to mediate the clustering of receptors and ion channels, especially at the high densities found in synapses (Hall and Sanes, 1993). Intriguingly, if the different members of the PSD-95 family were to have distinct channel binding specificities in vivo, heteromerization between them would increase the variety of ion channels in a cluster. Indeed, we have recently isolated a novel protein that interacts relatively specifically with chapsyn-110 (M. S., unpublished data), consistent with the idea that heteromultimerization of chapsyn-110 and PSD-95 could regulate the heterogeneity and composition of membrane protein clusters. In these heterogeneous clusters, the stoichiometric ratio of different classes of ion channel could be altered simply by regulating the degree of heteromerization between chapsyn-110, PSD-95, and their relatives.

Experimental Procedures

Chapsyn-110 cDNA Cloning

The original human chapsyn-110 cDNA fragment was isolated in a yeast two-hybrid screen using Kv1.4 as bait ("clone 5" in Kim et al., 1995). The clone 5 fragment was used as a hybridization probe to obtain from a human lambda gt11 human brain cDNA library (Clontech) further cDNAs that encompassed residues 117-870 of human chapsyn-110 . Clones containing sequences upstream of amino acid 117 were obtained by 5' RACE using "Marathon"-ready human brain cDNA (Clontech).

The rat chapsyn-110 cDNA was isolated after screening a rat brain cDNA lambda gt11 library (Clontech) with radiolabeled PSD-95 cDNA (Cho et al., 1992). ~300,000 plaques were screened at low stringency conditions (Church and Gilbert, 1984). A 1.6 kb EcoRI

fragment from one of the positives was subcloned in Bluescript KS-plasmid and used to screen 300,000 plaques from the same library at high stringency (Church and Gilbert, 1984). We identified and analyzed six positives further, and used two cDNA clones to generate the 3 kb cDNA, which contains the whole coding region.

Northern Analysis

Total RNA was isolated from various tissues as described by Chomczynski and Sacchi (1987). 10 μg of total RNA was separated on 1.5% agarose formaldehyde gels, transferred to Hybond nylon membrane (Amersham), and UV-crosslinked. A 1.6 kb PCR product (nucleotide 800-2,400) was amplified using the rat chapsyn-110 cDNA as template, and then used to generate random primed radiolabeled probe.

Antibodies

Anti-chapsyn-110 antisera were raised by immunizing rabbits with a hexahistidine-tagged fusion protein incorporating residues 343-660 of human chapsyn-110 (Figure 1A, diagram). This segment of the chapsyn-110 cDNA was amplified by PCR and subcloned in frame in the pRSETB vector (Invitrogen). The resulting bacterial fusion protein was purified to homogeneity by Nickel-NTA affinity chromatography. To obtain chapsyn-110-specific antibodies that did not cross-react with PSD-95 or SAP97, the antisera were affinity purified using two thioredoxin fusion proteins encoding unique segments of chapsyn-110 (see Figure 1B): Chapsyn-S23 (incorporating residues 343-417), and Chapsyn-INS (incorporating residues 627-660). Plasmid constructs were prepared by PCR amplification of the desired cDNA segment and subcloning into pET TRX (thioredoxin) fusion system vector 32 (Novagen), which also includes a hexahistidine segment, thus allowing purification of the resulting fusion proteins by Ni-NTA affinity chromatography. Purified Chapsyn-S23 and Chapsyn-INS fusion proteins were coupled to separate columns (Sulfolink, Pierce) for affinity purification of the respective antibodies.

SAP97 antisera were generated by immunizing rabbits with a GST fusion protein (constructed in pGEX4T-1, Pharmacia) incorporating residues 1-105 of rat SAP97, a unique region of the SAP97 protein (see Figure 1). Antibodies were then affinity purified using a purified thioredoxin fusion of the same SAP97 segment (constructed in pET TRX 32, Novagen), coupled to Sulfolink columns (Pierce).

PSD-95-specific antibodies used in immunohistochemistry, immunofluorescence, and immunoprecipitation were generated in guinea pig, and have been described in Kim et al., 1995. For PSD-95 immunoblotting, we used affinity purified rabbit anti-peptide antibodies raised against the synthetic peptide CSKRRVERREWSRLKA KDWG, corresponding to amino acids 490-508 of PSD-95. The N-terminal cysteine residue was added for coupling purposes.

Anti-NR1 mouse monoclonal antibody was purchased from Pharmingen. NR2A or NR2B antibodies have been described in Sheng et al., 1994a.

Immunoblotting, Immunohistochemistry, and Immunoprecipitation

Cell lysates from transfected COS-7 cells were prepared by Dounce homogenization in phosphate buffered saline (PBS) containing protease inhibitors. Crude synaptosomal membrane fractions from rat brain (Sheng et al., 1992), and detergent-extracted postsynaptic density (PSD) fractions (Cho et al., 1992) were prepared as previously described. Immunoblotting with enhanced chemiluminescence reagents (ECL, Amersham) was performed as described (Sheng et al., 1993, 1994b), except that semi-dry electrophoretic transfer of the proteins to nitrocellulose was used. Affinity purified antibodies were used at ~1 μg/ml concentration.

Immunohistochemistry was performed on Vibratome-cut 50 μm floating brain sections from Sprague Dawley rats (~8 weeks age) perfused transcardiacally with 4% paraformaldehyde, and permeabilized with either 0.3% Triton X-100 or 50% ethanol, as described in Sheng et al. (1994b), and Liu et al. (1994). Affinity purified antibodies were used at 0.5-1 μg/ml, final concentration.

Coimmunoprecipitation was performed as described in Sheng et al. (1994a). Dounce homogenized lysates from transfected COS-7 cells were extracted in 50-fold volume of Tris-buffered saline (TBS,

pH 7.4) containing 1% NP-40, 0.5% deoxycholate, 0.1% SDS at 4°C for 1 hr. After centrifugation at 16,000g for 30 min, the supernatant was incubated with affinity- purified primary antibody (2 μg/ml) at 4°C for 1 hr. Immunoprecipitates were collected with protein A-sepharose, separated in a 6% SDS PAGE gel and analyzed by immunoblotting.

DNA Constructions

For expression in COS-7 cells, Kv1.4, PSD-95, SAP97, chapsyn-110, NR1 (Moriyoshi et al., 1991), NR2A and NR2B (Ishii et al., 1993; Monyer et al., 1992) cDNAs were subcloned in the mammalian expression vector GW1-CMV (British Biotechnology). The -ETDA mutation of the C-terminus of Kv1.4 was generated by PCR amplification of the entire Kv1.4 coding region, using mutant 3′ primer that substituted A for V at the C-terminal residue. The resulting product was subcloned into GW1-CMV expression vector, like the wild-type Kv1.4. For myc-tagging of chapsyn-110, an AscI site was introduced between amino acid 14 and 15 in the chapsyn-110 cDNA by inverse PCR, and a cassette encoding the myc epitotpe (EQKLISEEDL) was inserted into the AscI site. Constructs were verified by nucleotide sequencing.

Cell Transfection and Coclustering Assay

COS-7 cells were transfected at ~40%-60% confluence, using the Lipofectamine method (GIBCO-BRL), either on poly-lysine coated microwell slides or coverslips (for coclustering experiments) or in 100 mm tissue culture dishes (for preparation of extracts for immunoblotting and immunoprecipitation). For transfection of NR1 and NR2A/NR2B subunits, a DNA mass ratio of 1:8 was used. Cells transfected with NMDA receptor subunits were incubated in a medium containing 0.5 mM ketamine. To assay clustering, cells were fixed 2 days after transfection in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with primary antibodies at 1 μg/ml concentration, and with Cy3- or FITC- labeled secondary antibodies (Jackson Immunoresearch) at dilutions of 1:1000 and 1:200, respectively. Immunofluorescence was viewed with a Zeiss Axioskop microscope.

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